

Biosynthesis of Dimethylnitrosamine in
Dimethylamine-Treated Mice After
Exposure to Nitrogen Dioxide

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Biosynthesis of Dimethylnitrosamine in Dimethylamine-Treated Mice After Exposure to Nitrogen Dioxide^{1,2,3}

EPA-600/J-81-609
JOURNAL ARTICLE

Zafar M. Iqbal,⁴ Kristine Dahl,⁴ and Samuel S. Epstein^{4,5}

ABSTRACT—These studies demonstrate the nitrosating potential of NO₂ in vivo in ICR mice. Groups of mice were gavaged with 2 mg dimethylamine (DMA) and exposed to NO₂ at levels from 0.04 to 44.5 ppm for periods up to 4 hours. Mice were individually frozen and blended to a powder, aliquots of which were homogenized in ice-cold dichloromethane and 35% aqueous methanol. Concentrates of organic extracts were analyzed for dimethylnitrosamine (DMN) by a Thermal Energy Analyzer with a gas chromatograph interface. Biosynthesis of DMN was dose- and time-dependent with relation to NO₂ exposure, reaching a maximum yield of 60–70 ng/mouse (0.0034% DMA conversion) at 2 hours. DMN biosynthesis was inhibited by sodium ascorbate and, more effectively, by ammonium sulfamate.—JNCI 1981; 67:137–141.

Nitrogen oxides are common atmospheric pollutants, largely resulting from the combustion of fossil fuels and a wide range of high temperature combustion processes, such as welding and foundry work (1). Additional sources of nitrogen oxides include mainstream and side-stream tobacco smoke (2–4) and domestic gas stoves (5, 6).

Chemical studies in the liquid or solid phase (7–10) and in the gas phase (11–17) and in vitro studies in plasma (18) and lung homogenates (19) have shown that NO₂ can nitrosate secondary amines, resulting in formation of carcinogenic nitrosamines. Extensive information is available on nitrosation of secondary and tertiary amines by sodium nitrite in chemical, in vitro, and in vivo systems (20–22), with recent findings on nitrosamine formation in humans (23, 24).

We have recently demonstrated in vivo nitrosating potential of NO₂ in mice, following gavage with morpholine (25). As an extension of these studies, we present here data on in vivo nitrosation of DMA in mice by inhaled NO₂.

MATERIALS AND METHODS

Analytic grade (>99.8% pure) DMA·HCl and nitrosamine standards, DMN and DPN, were purchased from Aldrich Chemical Co., Milwaukee, Wis. The following chemicals and reagents were purchased: pesticide-grade methanol from Fisher Scientific Co., Fair Lawn, N.J.; glass-distilled dichloromethane from Burdick & Jackson Laboratories Inc., Muskegon, Mich.; sodium ascorbate and ammonium sulfamate from Sigma Chemical Co., St. Louis, Mo.; and liquid nitrogen from Liquefied Industrial Gases, Inc., Hillside, Ill. Tanks of custom-grade, certified-standard NO₂ at specified concentrations were purchased from Matheson Co., Inc., Joliet, Ill. Dynacal Permeation Devices (standard- and low-emission and wafer devices) were

obtained from Metronics, Santa Clara, Calif. Ex-tubes were purchased from Analytichem International, Lawn-dale, Calif.

Male ICR mice (25–30 g; 6 wk old) were purchased from Scientific Small Animals Farm, Arlington Heights, Ill., and maintained on Purina Chow pellets for up to 1 week. Mice were starved in a wire-bottom cage for 16–18 hours prior to test.

Nalgene vacuum desiccators (Nalge 5310), purchased from Scientific Products, Inc., McGaw Park, Ill., were modified for use as exposure chambers with gas inflow from the bottom and exhaust from the top. Groups of 3–4 mice were put on a wire grid placed over a perforated desiccator plate to prevent the reingestion of any urine or feces excreted during exposure. The desiccators were sealed with vacuum grease before NO₂ exposure.

A TEA (Model 502; Thermo Electron Corp., Waltham, Mass.) interfaced to a Model 661 gas chromatograph (Thermo Electron Corp.) was used for nitrosamine analysis. A stainless-steel column (14 feet × 1/8 inch) packed with 10% Carbowax 20M plus 0.5% KOH on Chromosorb WHP 80/100 was used under isothermal conditions at 175°C. The argon carrier gas achieved a flow rate of 22 ml/minute at an inlet pressure of 60 pounds per square inch. Concentrated samples of DCM and methanol, DCM extracts of distilled water, and all aqueous solutions were checked for contamination with DMN and other nitrosamines; all solvents and solutions were nitrosamine-free (detection limit, 0.1 ppb).

NO₂ exposure.—For exposure to 41.5 or 44.5 ppm NO₂, gas from stock tanks was directly introduced into the exposure chambers from which it was passively exhausted into a chemical hood. NO₂ levels from 8 to 25 ppm were diluted by the mixture of stock NO₂ (41.5

ABBREVIATIONS USED: DCM=dichloromethane; DMA=dimethylamine; DMN=dimethylnitrosamine; DPN=di-n-propylnitrosamine; TEA=Thermal Energy Analyzer.

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⁴ School of Public Health, University of Illinois at the Medical Center, P.O. Box 6998, Chicago, Ill. 60680.

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or 44.5 ppm) with air at appropriate flow rates to reach the required dilutions. For 0.04–3 ppm NO₂ levels, Dynacal Permeation Devices were used as follows: 0.04 ppm (low-emission tube at 25°C; airflow, 3 liters/min); 0.1 ppm (wafer device at 25°C; airflow, 3 liters/min); 0.75 ppm (standard-emission 2-cm tube at 30°C; airflow, 2.5 liters/min); and 3 ppm (standard-emission 10-cm tube at 29°C; airflow, 2.5 liters/min). All permeation devices were conditioned overnight before use (25). The accuracy of gas mixtures was checked by periodic monitoring and NO₂ analysis in the chamber exhaust, with the use of the Griess-Saltzman colorimetric assay with a fritted bubbler sensitive to 0.005 ppm NO₂ (26).

DMN biosynthesis.—Groups of 3–4 mice were gavaged with freshly prepared solutions of 2 mg DMA in 0.25 ml distilled water and immediately placed in an exposure chamber; this concentration of DMA was selected on the basis of preliminary range-finding studies. Mice were exposed to NO₂ (5 ft³/hr; 20-vol changes/hr) under the following conditions: at 41.5 ppm NO₂ for 0.5, 1, 2, 3, and 4 hours and at 0.04, 0.1, 0.75, 3, 8, 15, 25, and 44.5 ppm NO₂ for 2 hours. Concurrent controls consisted of groups of 3 mice exposed in separate chambers to 41.5 ppm NO₂ for periods between 0.5 and 4 hours and to the specified NO₂ concentrations between 0.04 and 44.5 ppm NO₂ for 2 hours; additional controls were groups of 3–4 mice gavaged with 2 mg DMA in 0.25 ml distilled water or 0.25 ml distilled water alone, followed by exposure to air for identical periods. After exposure, mice were individually frozen in liquid nitrogen and blended to a powder before DMN analysis (25).

Inhibition of DMN biosynthesis.—Groups of 3–4 mice were gavaged first with 2 mg DMA in 0.25 ml distilled water and then with aqueous solutions of 50–250 mg sodium ascorbate or 10–100 mg ammonium sulfamate. They were then exposed for 2 hours to NO₂ at 41.5 ppm (ascorbate-fed groups) and 44.5 ppm (sulfamate-fed groups). Control mice were fed DMA and equivalent volumes of distilled water (instead of inhibitors) and were exposed in the same manner. Additional controls were exposed to DMA alone and NO₂ alone.

In a parallel experiment, groups of mice were gavaged with 2 mg DMA, then exposed to 41.5 or 44.5 ppm NO₂ for 2 hours, frozen in liquid nitrogen, and blended to a powder. Prior to DMN analysis, 8-g powder aliquots of each mouse were mixed with aqueous solutions of 50–250 mg sodium ascorbate, 50–100 mg ammonium sulfamate, or water alone.

DMN analysis.—DMN and other nitrosamines were quantitatively determined in frozen mouse powders (25). Two or three aliquots of approximately 8 g were taken from each mouse powder and blended with 75 ml of ice-cold 35% aqueous methanol in a Waring Blendor (5 min; low speed); a known amount of DPN (240–260 ng) was then added, and blending was continued for 1–2 minutes. Homogenates were halved and centrifuged (4,200×g, 25 min at 5°C, swinging bucket), supernatants removed, and the pellets extracted twice with an equal volume of DCM (total, 180 ml). The

organic layer was dried by passage through an unbuffered Ex-tube and concentrated to 2 ml in a Kuderna Danish concentrator (Kontes, 250 ml) in a 65°C bath. Aliquots (20 µl) of the concentrates from each of two or three powder samples were injected into the TEA-gas chromatograph for nitrosamine analysis. Peaks were identified and quantitated by comparison with the retention time and response of reference DMN. Values are corrected for any background control DMN levels and for DPN-standard recoveries.

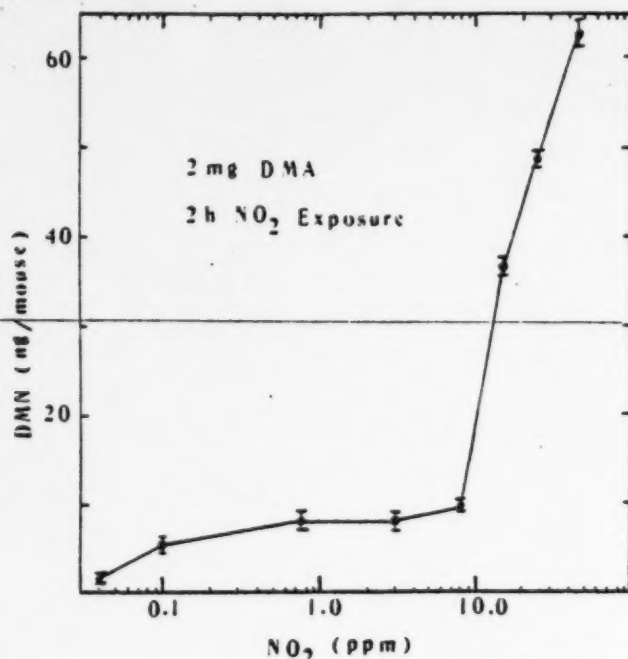
RESULTS

All aqueous solutions (DMA, sodium ascorbate, and ammonium sulfamate), distilled water, and solvents used in these experiments were free of DMN and other nitrosamines (detection limit, 0.1 ppb). Low levels of DMN were observed in various control mice, with the following values (nanograms) per mouse: 5.6–9.1 (NO₂ alone), 9.3–11.9 (DMA alone), and 5.1–7.3 (water alone). No other nitrosamines were detected. DMN levels observed in DMA control groups were subtracted from the values in each experimental group. Recovery of the DPN internal standard from mouse powder ranged from 85 to 94%; all DMN values were corrected for DPN recovery. DMN and DPN values per gram mouse powder varied less than 5% from the average. Preliminary range-finding experiments over a range from 0.5 to 4 mg DMA demonstrated that DMN yields were maximum in mice gavaged with 2 mg DMA, followed by exposure to 43 ppm NO₂ for 1 hour, 54 ppm NO₂ for 1 hour, or 49 ppm NO₂ for 4 hours.

DMN biosynthesis was dependent on NO₂ concentrations (text-fig. 1). When mice gavaged with 2 mg were exposed to 0.04 ppm NO₂ for 2 hours, DMN levels were increased only minimally over control values. At higher NO₂ levels, DMN yields were significantly increased ($P < 0.01$) over 0.04 ppm NO₂ or control values. From 0.1 to 8 ppm NO₂, yields of DMN were approximately 10 ng/mouse. From 8 to 44.5 ppm, yields increased linearly, reaching a maximum of 60–70 ng DMN/mouse, equivalent to 0.0034% conversion of DMA.

DMA biosynthesis was time-dependent with relation to NO₂ exposure (text-fig. 2). When mice gavaged with 2 mg DMA were exposed to 41.5 ppm NO₂ for 0.5 hour, approximately 20 ng DMN/mouse was detected, which was significantly higher ($P < 0.01$) than the DMN level in controls. An increase in NO₂ exposure from 0.5 to 1 hour resulted in only a minimal increase in DMN yields. Maximum yields (60–70 ng/mouse) were obtained following a 2-hour exposure to NO₂; extending exposure to 4 hours resulted in an apparently paradoxical decrease to less than 20 ng DMN/mouse.

DMN biosynthesis was inhibited by sodium ascorbate (text-fig. 3) and ammonium sulfamate (text-fig. 4). When mice were gavaged successively with 2 mg DMA and 50–250 mg sodium ascorbate, before a 2-hour exposure to 44.5 ppm NO₂, DMN levels were significantly lower ($P < 0.01$) than those in ascorbate-free controls; inhibition ranged from 54% (50 mg ascorbate)

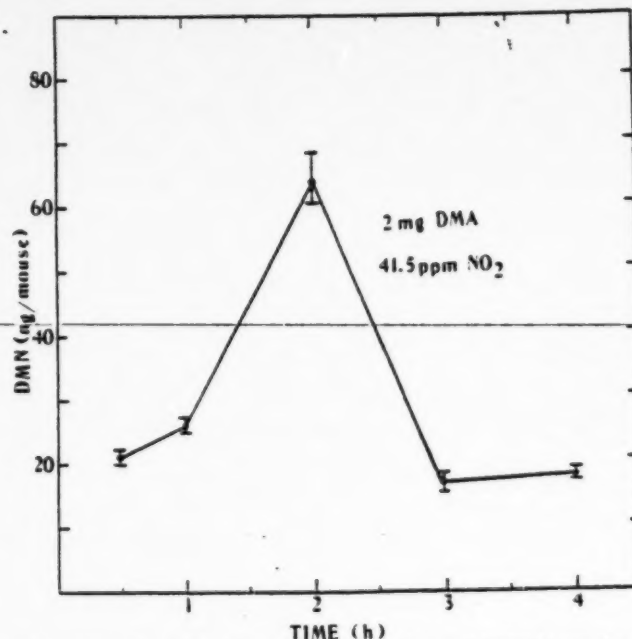


TEXT-FIGURE 1.—NO₂-concentration dependence of DMN biosynthesis in mice. Groups of 3–4 mice were gavaged with 2 mg DMA (0.25 ml distilled water) and immediately transferred to chambers for a 2-hr exposure to 0.04, 0.1, 0.75, 3, 8, 15, 25, and 44.5 ppm NO₂. For generation of 0.04–3 ppm NO₂, Dynacal Permeation Devices were used. For 8–25 ppm NO₂, stock tank (44.5 ppm) NO₂ was diluted with air; 44.5 ppm NO₂ was used directly from tanks. Mice were frozen and analyzed for DMN, as described in "Materials and Methods;" background DMN levels found in DMN controls (9.3–11.9 ng) were subtracted from all experimental values that represent an average of 3–4 mice $\pm$ SD ($\bullet$).

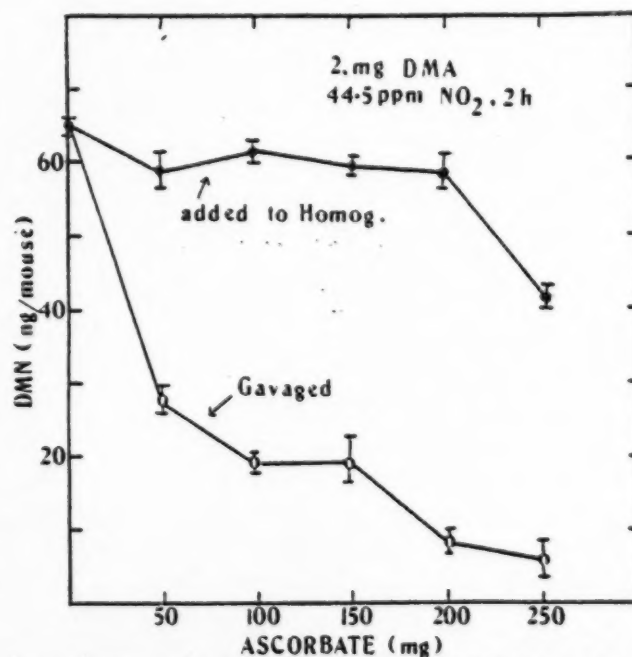
to 92.3% (250 mg ascorbate). However, when 50–200 mg ascorbate was added to the homogenates of the exposed mice (2 mg DMA; 44.5 ppm NO₂, 2 hr), the DMN yields decreased only 5–7% in relation to yields in ascorbate-free homogenates. Addition of 250 mg ascorbate to homogenates resulted in a substantial (35.4%) decrease, which was also accompanied by a significantly lower (75%) recovery of the internal standard DPN. Feeding of ammonium sulfamate (50–100 mg) appeared to be a more effective inhibitor of DMN biosynthesis, averaging between 93 and 97% inhibition, but ammonium sulfate was highly toxic when fed at levels above 100 mg. Addition of 50–250 mg sulfamate to homogenates of exposed mice (2 mg DMA; 41.5 ppm NO₂, 2 hr) did not result in any significant decrease in DMN.

DISCUSSION

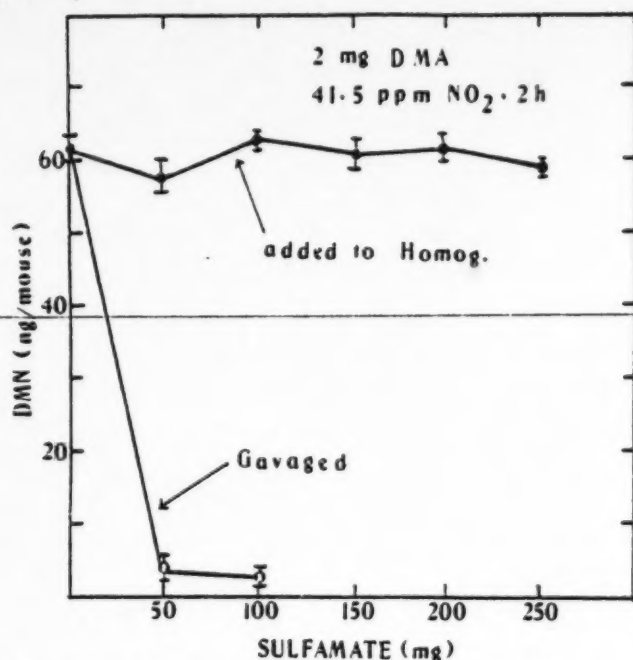
With the exception of earlier studies on NO₂-induced nitrosation of morpholine in mice (25), *in vivo* nitrosation of amines by inhaled NO₂ has not been previously demonstrated. The present studies demonstrate the *in vivo* nitrosating potential of NO₂ in mice gavaged with



TEXT-FIGURE 2.—NO₂ exposure time dependence of DMN biosynthesis in mice. Groups of 3–4 mice were gavaged with 2 mg DMA and exposed to 41.5 ppm NO₂ for 0.5, 1, 2, 3, and 4 hr.



TEXT-FIGURE 3.—Sodium ascorbate inhibition of DMN biosynthesis in mice gavaged with DMA and exposed to NO₂. Groups of 3–4 mice were gavaged with 2 mg DMA, immediately followed by sodium ascorbate (50–250 mg) and then exposed to 44.5 ppm NO₂ for 2 hr ($\circ$). Groups of 3–4 mice were gavaged with 2 mg DMA and exposed to NO₂ (44.5 ppm) before freezing; aqueous solutions containing 50–250 mg sodium ascorbate were added to aliquots (8 g) of mouse powder and homogenized ($\bullet$).



TEXT-FIGURE 4.—Ammonium sulfamate inhibition of DMN biosynthesis in mice gavaged with DMA and exposed to 41.5 ppm NO_2 . DMA-fed mice were gavaged with 50–100 mg ammonium sulfamate, followed by NO_2 exposure (○); in other groups 50–250 mg ammonium sulfamate was added to powders of mice exposed to DMA and NO_2 (●).

DMA. The resultant DMN biosynthesis was dependent on NO_2 concentration and duration of exposure. The decrease in DMN levels observed at 3 and 4 hours in the time-dependent experiments (text-fig. 2) may be due to competing factors such as DMN metabolism and excretion or macromolecular binding, including possible removal of DMA itself. Possible artifactual formation of DMN during the analytic procedure appears to range between 5 and 10%. Low levels of DMN in different controls (up to 12 ng/mouse) may reflect DMN contamination of feed (controls given water alone), nitrosation of endogenous DMA by NO_2 (controls given NO_2 alone), or nitrosation of endogenous and exogenous DMA by ambient NO_2 and nitrite sources (controls given DMA alone); however, the control DMN levels were subtracted from experimental values. DMN biosynthesis was inhibited by feeding with sodium ascorbate or ammonium sulfamate. Our data do not, however, identify the site of nitrosation or its mechanism.

Liquid-phase nitrosation of amines by NO_2 is believed to be mediated by the reactive forms of N_2O_3 or N_2O_4 or by a free radical process (7–10). Nitrosation of DMA in vivo could occur in the pulmonary capillaries or in the systemic bloodstream by the nitrosating radical formed after absorption of NO_2 ; nitrogen oxides from sources such as tobacco smoke are known to be readily absorbed into the blood during inhalation (1, 2). Nitrous acid, formed as a result of NO_2 inhalation,

could be secreted into saliva and then react with DMA in the stomach.

The possibility of in vivo nitrosation of amines by nitrogen oxides has clear public health implications (1–6). Current ambient and occupational standards for NO_2 are 0.05 ppm ($100 \mu\text{g}/\text{m}^3$) for an annual average and 5 ppm for a 15-minute ceiling value, respectively. A short-term NO_2 standard, although mandated by the August 1977 amendments to the Clean Air Act (section 109c and d; 42 the U.S. code, section 7409 et seq) has not yet been promulgated. The amendments also specifically required revision of the Air Quality Criteria Document to address NO_2 derivatives including nitrosamines. Human exposure to nitrogen oxides is variable, occurring at levels up to 0.4 ppm in the U.S. urban ambient air and up to 1,600 ppm in mainstream tobacco smoke (2–4). Limited epidemiologic studies have suggested the existence of some relationship between ambient NO_2 concentrations and an urban excess of cancer (27, 28). Speculation has also been made as to the possibility of an NO_2 -nitrosamine-cancer link (29). Our studies demonstrate the possible hazards due to in vivo NO_2 -mediated nitrosation of DMA, an amine found both endogenously and exogenously in common sources such as meat and fish.

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